OIL BLUE NA AS A STAIN FOR RUBBER IN SECTIONED OR GROUND PLANT TISSUES[1]

ROBERT T. WHITTENBERGER, *Eastern Regional Research Laboratory*[2], *Philadelphia, Pennsylvania*

ABSTRACT.—**Oil blue NA (Calco), a stain which colors rubber bright blue, has been used effectively in studying the distribution of rubber in several plant species. Fresh or fixed sections are cut, bleached with Javelle water or NaOCl solution, treated with 9% KOH in 95% ethanol, washed with several changes of water and finally with 95% ethanol, and stained with 0.05% oil blue NA in 70% ethanol. Sections are rinsed in 50% ethanol, placed in 40% glycerin, and mounted in glycerin jelly.**

For the detection of changes in the distribution and character of rubber in milled or ground tissues, much the same staining procedure is followed. The stained tissues usually are examined and dissected under a stereoscopic microscope, a procedure which permits rubber to be recognized by both its staining reaction and by a more specific property, elastic elongation.

A microscopic technic is presented whereby it is possible to determine approximately the relative proportion of dispersed and coagulated rubber latex in unstained tissues.

In studies on rubber-bearing plants, histologists (Lloyd, 1911; Hall and Goodspeed, 1919; Spencer, 1939; Artschwager, 1943) have utilized stains (alkanet, Sudan III, Sudan IV) which impart a red, orange, or yellow color to rubber. There is now available, however, a stain, oil blue NA (Calco), which colors rubber clear, bright blue. For some types of histological work, especially with plants having natural yellow and reddish pigments, the blue stain is superior to the red.

Oil blue NA[3] is an oil-soluble stain reported to be 1,4 bis amylamino anthraquinone. That the commercial product may contain a minor impurity is suggested by the fact that under certain condi-

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ditions, as when a large excess (0.5%) of the powder is mixed with 50% ethanol, a purple rather than a blue solution is obtained. Washing the powder several times with 50% ethanol selectively removes the more soluble purple component. In the present paper, concentrations of the stain which color rubber blue rather than purple are described.

Like other rubber stains, oil blue NA is not specific for rubber but also stains other plant constituents, such as suberin, cutin, oils, fats, and resins. These materials, however, either can be removed prior to or during staining, or else can be recognized, with the possible exception of resin, by one having a knowledge of plant anatomy and cytology.

The technic to be used for staining rubber in plant tissues depends on the character of the sample and the information sought. The technics presented in this paper have been tested and used effectively at this laboratory in developing methods for the recovery of rubber from plants. We have been interested principally in the distribution and character of rubber in untreated plants and in tracing the changes effected by various chemical and mechanical treatments accessory to recovery or analytical procedures.

Staining of Sections.—The staining technic outlined below has given good results with *Parthenium argentatum* (guayule), *Taraxacum koksaghyz* (Russian dandelion), *Chrysothamnus nauseosus* (rabbit brush), *Actinea Richardsoni* (Colorado rubber plant or pingue), *Cryptostegia grandiflora* and hybrid, and *Landolphia Thollonii.* Sections usually are prepared by sectioning frozen plant material (fixed or unfixed) previously infiltrated under suction with 5% warm gelatin. In some cases, embedding herbaceous and semi-woody tissues in Carbowax 4000 in a manner described by Richards et al. (1942) diminishes the amount of displacement of rubber during sectioning. Treatments (such as paraffin embedding) which dissolve rubber preliminary to sectioning should be avoided.

1. The sections are bleached with 1 ml. of Javelle water or NaOCl solution (containing 5% available chlorine) for 5 minutes at 25° C. in a closed container.
2. To this is added 2 ml. of 9% KOH in 95% ethanol which is permitted to act for 30 to 40 minutes at 25° C. (or for a shorter period at a higher temperature).
3. The mixed solutions are removed from the sections, which are then washed several times with water and finally with 95% ethanol.
4. The sections (in a closed container) are stained for approximately one hour with 0.05% oil blue NA in 70% ethanol.

5. The sections are washed in 50% ethanol for a few seconds, placed in 40% glycerin for a few minutes, and then mounted in glycerin jelly.

Cell or duct inclusions which take the blue stain are usually rubber, since non-rubber substances which take the stain are in a large measure removed by the preliminary treatments. The Javelle water or NaOCl solution destroys protoplasm, clears the cells, and results in a brighter staining of the rubber, whereas the alcoholic KOH dissolves or saponifies suberin, cutin, oils, fats, resins and waxes (Miller, 1938; Rawlins, 1933; Sampson, 1931; Molisch, 1922). When there is any question as to the complete elimination of non-rubber fatty or resinous substances, a portion of the sections should be extracted before staining, some with hot acetone and others with hot benzene. Cell or duct inclusions which take the stain after extraction with acetone and which are removed by extraction with benzene are probably rubber. It should be noted that acetone may dissolve the low molecular weight fraction of rubber[4] and that another fraction may be insoluble in benzene (Memmler, 1934; Stevens, 1943). Sections prepared by the foregoing technic at this laboratory are well preserved and unfaded after eighteen months, during which time several of the slides were subjected repeatedly to microprojection.

Variations in Staining Procedure.—A convenient although perhaps less satisfactory technic than that outlined above for the staining of rubber in plant sections involves the use of a modified Amann's lactophenol. This is prepared by dissolving 0.08 g. of oil blue NA powder in 20 ml. of melted phenol, which is then mixed with 20 ml. of lactic acid, 40 ml. of glycerin, and 20 ml. of water. Extracted or unextracted, bleached or unbleached sections, after being dipped in 25% glycerin, are mounted in the lactophenol solution, which thus serves as a staining, clearing, and mounting medium. The mounts, of course, are temporary.

Another variation permits the staining of rubber simultaneously with the saponification treatment; 0.25% oil blue NA dissolved in the alcoholic KOH will quickly stain rubber, provided a bleaching agent is not present. After being stained, the sections are washed in 50% ethanol, dipped in 40% glycerin, and mounted in glycerin jelly.

Although counterstaining of non-rubber constituents can be accomplished simultaneously with the staining of rubber by mixing safranin, etc., with oil blue NA in step 4, this procedure has been

[4]Unpublished data, Eastern Regional Research Laboratory.

found to offer no advantage in the identification of rubber, since the contrast in brightness between rubber and non-rubber materials is diminished and the background is made more opaque. The bright blue color of the stained rubber can be more readily distinguished against a white background such as is obtained by bleaching the tissues. Counterstaining is desirable only if emphasis is to be placed on non-rubber constituents. In this event the bleaching and saponification treatments should be omitted, since they totally destroy the cytological detail of the non-rubber protoplasmic elements. For cytological studies fixing and bulk staining of tissues preliminary to sectioning may be desirable.

Staining of Ground Tissues.—For staining rubber in ground, milled, or incompletely extracted plant tissues, much the same procedure used for staining sections may be followed. Better results have been obtained, however, by using a weaker staining solution (0.02% oil blue NA in 55% ethanol) for a longer period (18 to 24 hours) and by increasing the initial bleaching period to 30 to 60 minutes, depending upon the nature of the plant material. A Hirsch funnel is convenient for the separation and washing of the tissues from the bleaching and saponifying solution. If permanent records are desired, the stained tissues can be mounted in glycerin jelly. Usually, however, they are examined under a stereoscopic microscope before mounting.

Examination of Ground Tissues.—In a few cases, such as in the examination of *Cryptostegia* leaf chlorenchyma, the compound microscope can be used advantageously for locating rubber in ground tissues. In the majority of cases, however, a wide-field stereoscopic microscope provides the more effective and rapid means. A larger quantity of material can be examined in a shorter time, thus making possible more accurate estimates of the amount of rubber present, and unbroken pieces of tissue can be oriented, dissected, and assayed for rubber.

After the short alcohol wash (step 5), the stained ground tissues are usually placed in water or glycerin in a Syracuse watch glass and examined under a stereoscopic microscope while being probed and manipulated with two needles. Rubber may be recognized not only by its staining reaction but also by a more specific property, namely, its elastic elongation (any dispersed latex rubber will have coagulated during the staining procedure, and thus be elastic). Even small amounts of rubber can be identified by these means, whether it occurs as thin films in individual guayule parenchyma cells or as traces in incompletely extracted kok-saghyz ducts. Indeed,

after some experience with samples of known rubber content, the small amount of rubber remaining in a ground sample that has been chemically analyzed for rubber (Spence and Caldwell, 1935) can be detected and the quantity estimated.

Determining the State of Rubber in Tissues.—It should be pointed out that staining cannot be used in studying all the problems arising in the microscopic examination of plant tissues for rubber. During a staining procedure, chemical and physical alterations are produced, including coagulation of any dispersed rubber latex. Only by direct microscopic observations on untreated living tissues is it possible to estimate the relative proportion of dispersed and coagulated rubber present. No other means is known to us for the approximate determination of this ratio, since chemical methods of analysis give information only on total rubber and mechanical treatment of tissues causes latex coagulation. The direct microscopic method described below as applied to guayule must be correlated with chemical analyses of the plant and with histological studies of stained sections.

Sections (or ground tissues), cut and kept under water or a latex-stabilizing solution, are dissected immediately under a stereoscopic microscope. When a latex-bearing cell is ruptured, a minute white cloud of latex is seen to issue from it if the latex is in the dispersed condition; if coagulation has occurred, the rubber of an individual cell can be stretched into a thin elastic strand. Dispersed latex can readily be distinguished from coagulated latex even in the same cell, and cells rich in rubber contrast sharply with cells of low rubber content. With surface illumination and dark background, cells containing dispersed latex appear opaque white; as latex coagulation occurs, they change to slightly translucent white, then to light yellow, and finally to brown. Of course, in chlorophyllous tissue, the color changes are modified. As a check upon the amount of coagulated latex remaining in a ruptured and crushed cell, it can be stained and re-examined, the stereoscopic and compound microscopes being used interchangeably. Under the compound microscope, dispersed latex in fresh, unstained tissues is distinguished by its Brownian movement.

After some experience with a given plant species, an histologist following the technic just described is able to make a fairly reliable estimate of both the condition and quantity of rubber even in unstained tissues. Such estimates have been of material value in developing large-scale methods for the recovery of dispersed rubber latex from both guayule and kok-saghyz, since they indicate the

effect of a recovery step on latex coagulation in tissues. The technic has been employed also in investigating the state of rubber in tissues of anatomically unfamiliar rubber-bearing plants. This knowledge is necessary for the intelligent planning of a recovery procedure, and may indicate whether the rubber can best be extracted by bleeding, solvents, retting, crushing, milling, or by some other means.

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